Cell Reports

Supplemental Information

Activity-Dependent Ubiquitination of GluA1 and GluA2 Regulates AMPA Receptor Intracellular Sorting and Degradation

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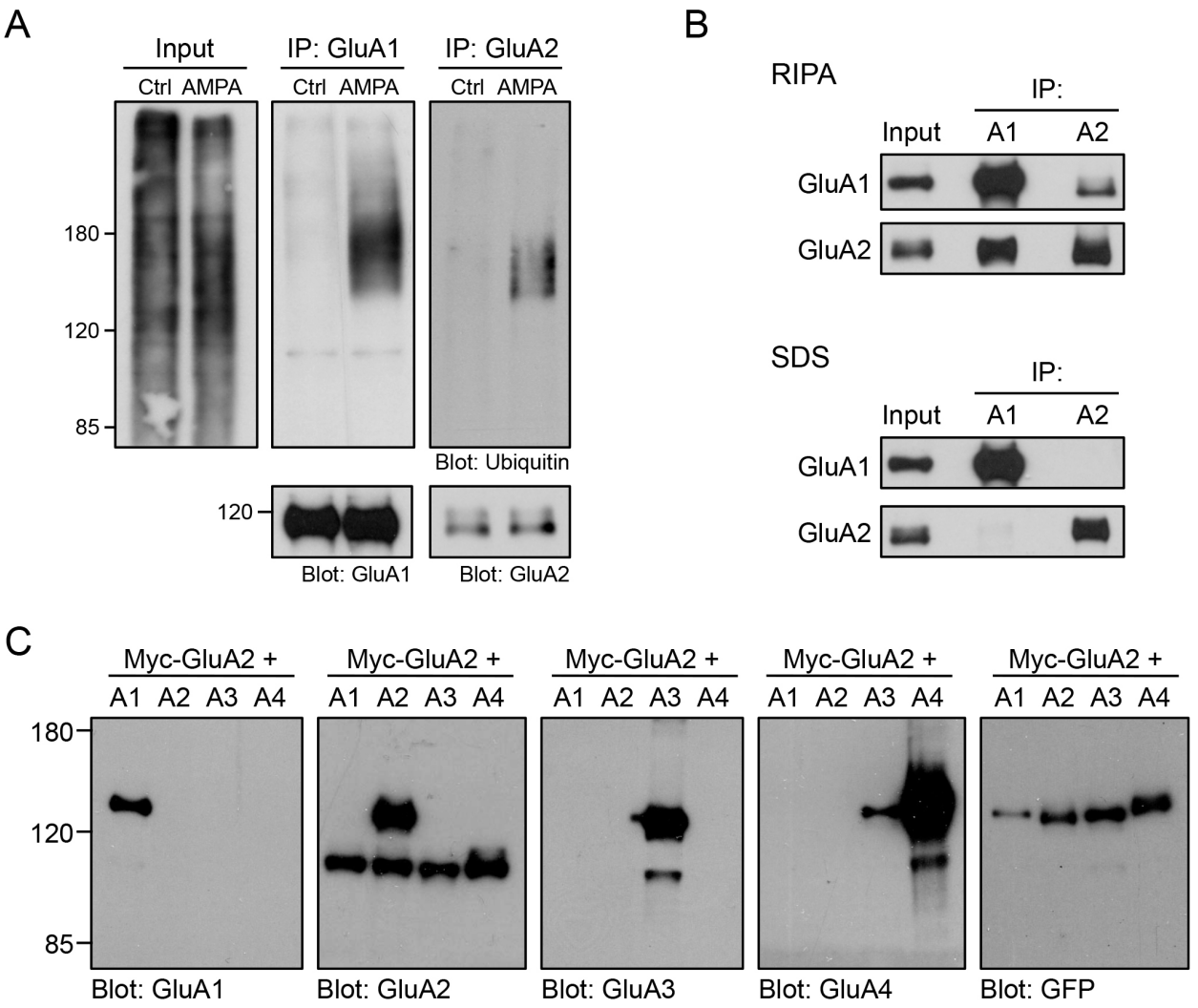

Figure S1, Related to Figure 1. Characterisation of the AMPA receptor ubiquitination assay and the specificity of AMPA receptor subunit antibodies.

(A) Cortical neurons were incubated in ACSF in the presence or absence of AMPA for 10 min at 37°C. Neurons were then lysed in 1% SDS and immunoprecipitated (IP) using anti-GluA1 or anti-GluA2 antibodies. Eluted proteins were subjected to western blot analysis and probed with anti-ubiquitin, anti-GluA1 and anti-GluA2 antibodies. (B) Cortical neurons were lysed in either RIPA buffer or 1% SDS, and immunoprecipitated with anti-GluA1 and anti-GluA2 antibodies. Bound proteins were eluted and probed with specific antibodies against GluA1 and GluA2 by western blotting analysis. (C) HEK293-T cells were co-transfected with myc-GluA2 and GFP-GluA1-4, then lysed and probed with specific antibodies against GluA1 (4.9D), GluA2 (6A), GluA3 (JH4300), GluA4 (JH4303) or GFP (JH4030) antibodies.

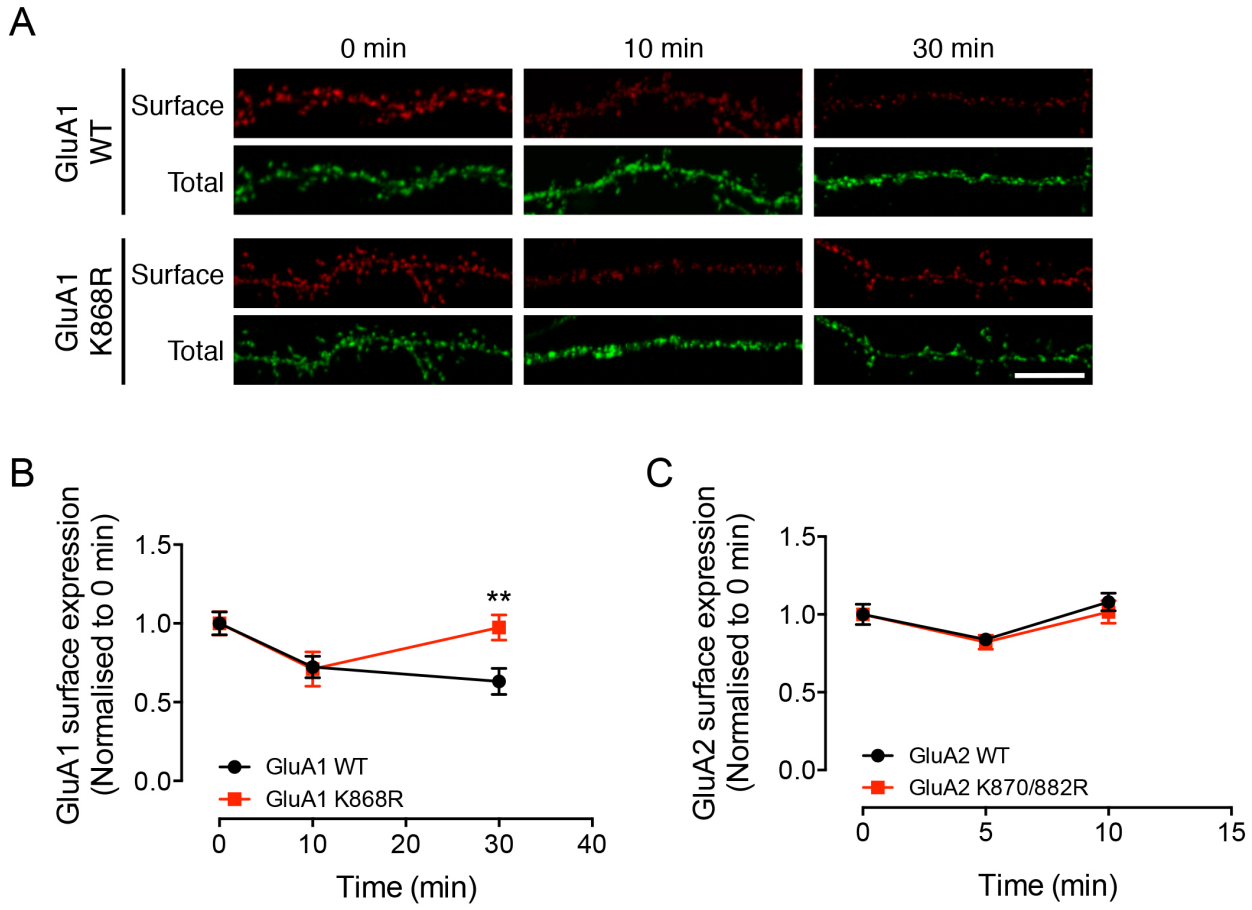

Figure S2, Related to Figure 5. Non-ubiquitinated GluA1 recycles back to the plasma membrane after ligand-induced endocytosis.

(A) Cultured hippocampal neurons were transfected with pH-GluA1, either wild-type (WT) or ubiquitin-deficient K868R mutant, at DIV 13. At DIV 15, surface pH-GluA1 was labelled with rabbit anti-GFP antibody for 15 min at 37°C. Representative images of total and surface GFP-GluA1 after AMPA treatment at various time points are shown (t = 0 min indicates cells not treated with AMPA). Scale bar, 10 µm. (B) Quantification of the surface/total pH-GluA1 ratio normalised to the value of control (t = 0 min) neurons. Data represent mean ± S.E.M (*t*-test, **p < 0.01; n = 14 neurons per group). (C) Quantification of the surface/total pH-GluA2 ratio normalised to the value of control (t = 0 min) neurons. Data represent mean ± S.E.M (n = 13 neurons per group).

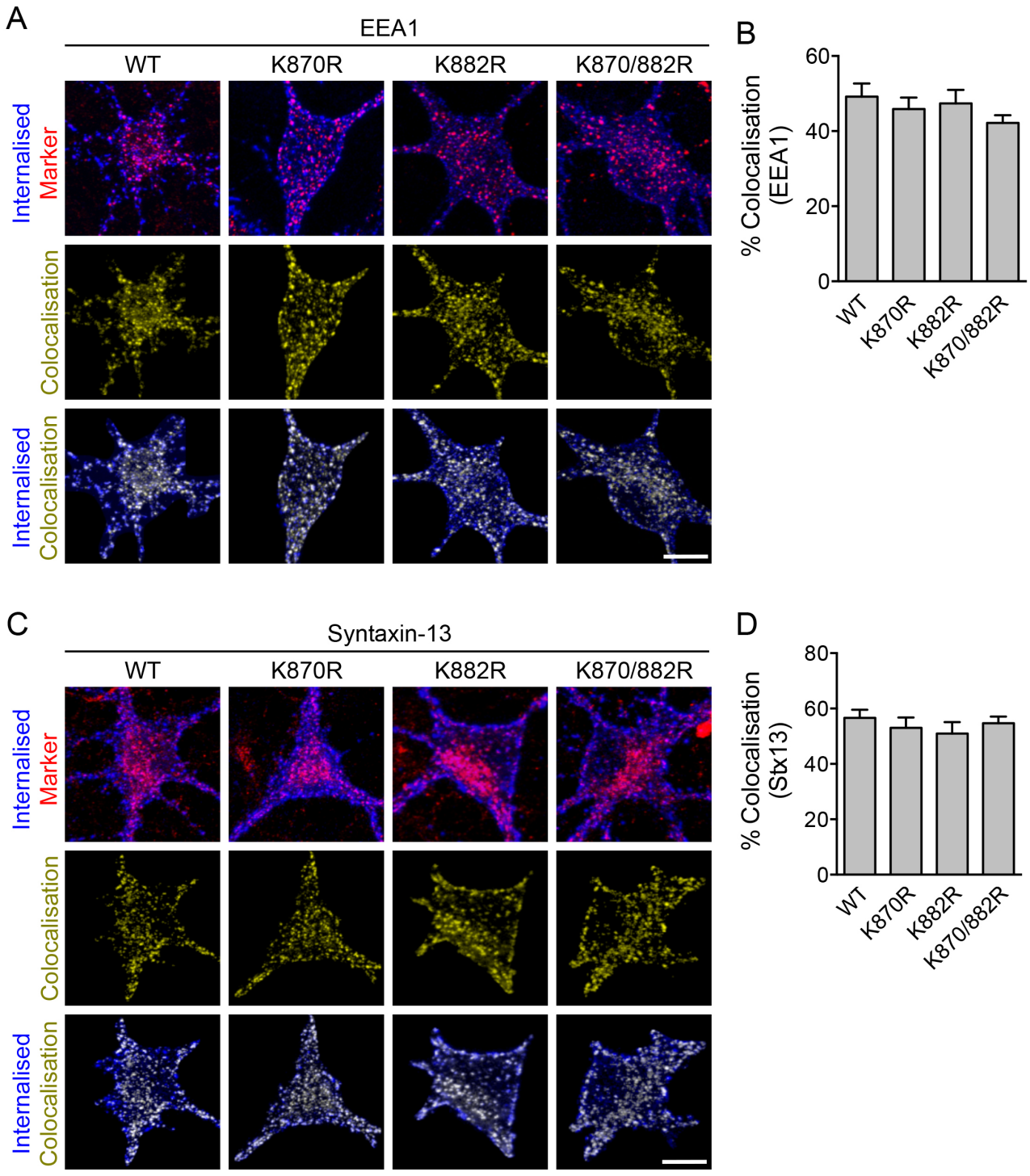

Figure S3, Related to Figure 5. Ubiquitination of GluA2 does not regulate post-endocytic endosomal sorting of receptors into early and recycling endosomes.

Hippocampal neurons were transiently transfected with pH-GluA2, either wild-type (WT) or ubiquitin-deficient mutants, at DIV 13 for 48 h. Live neurons were labelled with anti-GFP antibodies, stimulated with AMPA for 2 min and returned to growth medium for 3 min (early

endosomes) or 8 min (recycling endosomes) to allow for GluA2 internalisation (blue) and sorting into different endosomal compartments (magenta). Simultaneous staining for the early endosome marker, EEA1 (A) and the recycling endosome marker, syntaxin-13 (C) revealed extensive colocalisation (yellow) with internalised pH-GluA2 after AMPA application. (B, D) The extent of association of internalised pH-GluA2 with EEA1- (B) and Stx13-labelled compartments (D) was quantified by image analysis as the colocalised signal as a percentage of the total internalised receptor signal. Scale bar, 10 μm. Data represent mean ± S.E.M (n = 19 to 24 neurons per group).

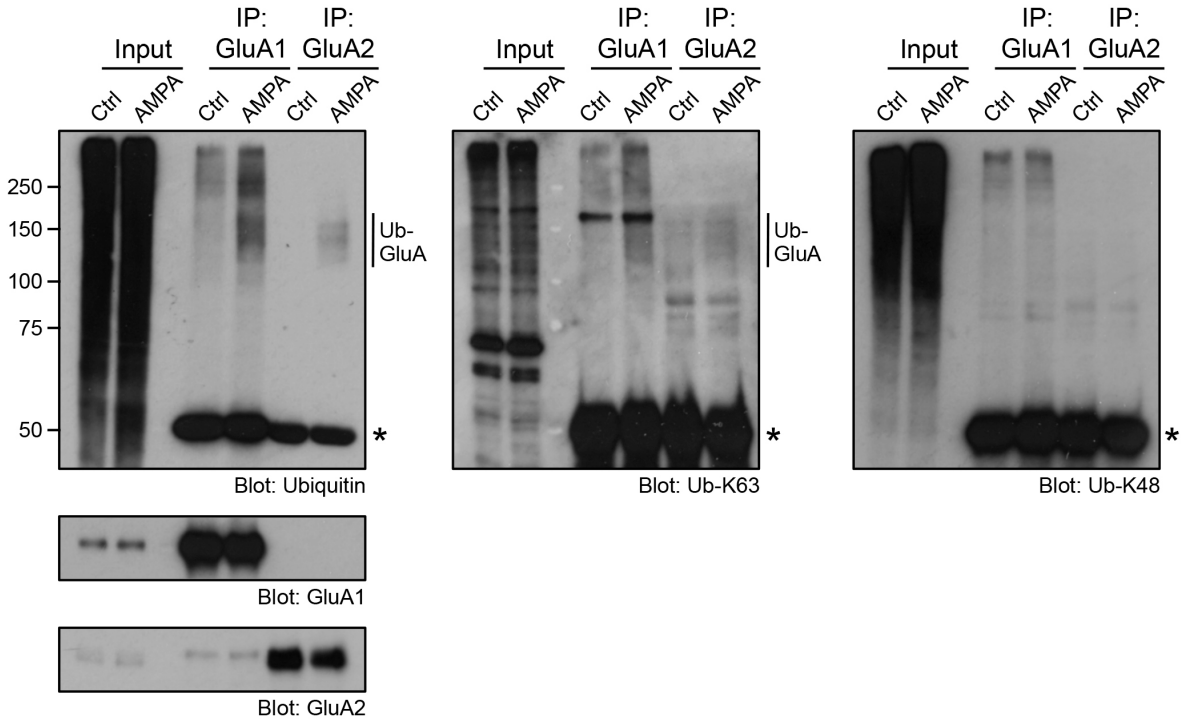

Figure S4, Related to Figure 5. The GluA1 and GluA2 subunits of AMPA receptors undergo K63-linked polyubiquitination.

Cortical neurons were incubated in ACSF in the presence or absence of AMPA for 10 min at 37°C. They were then lysed in 1% SDS and immunoprecipitated (IP) using anti-GluA1 or anti-GluA2 antibodies. Eluted proteins were subjected to western blot analysis and probed with anti-ubiquitin, anti-K63-linked polyubiquitin, anti-K48-linked polyubiquitin, anti-GluA1 and anti-GluA2 antibodies. Asterisks denote IgG heavy chains.

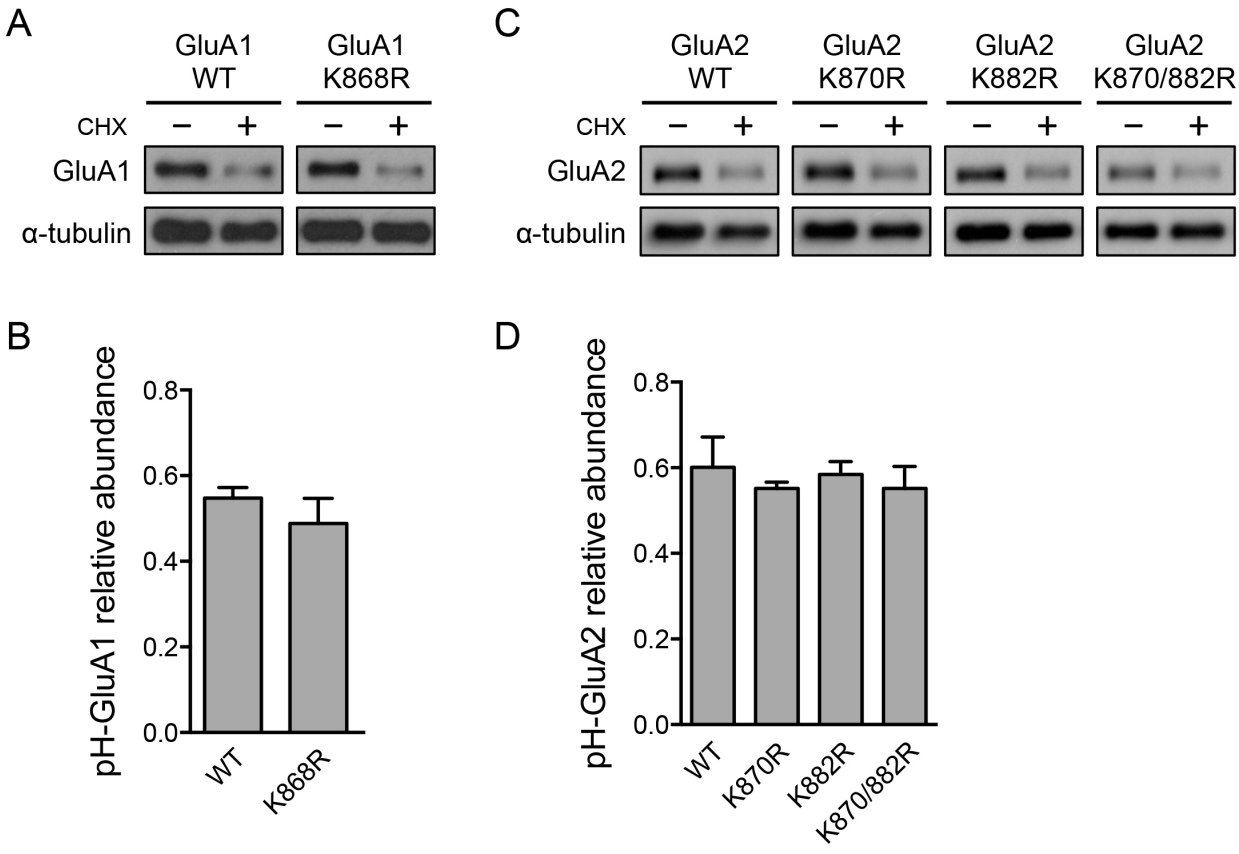

Figure S5, Related to Figure 6. Ubiquitination of GluA1 and GluA2 does not regulate basal protein turnover of AMPA receptors

(A−D) Cortical neurons were electroporated with pH-GluA1 (A) and pH-GluA2 (C) constructs, either wild-type (WT) or ubiquitin-deficient mutants as indicated, prior to plating. Neurons were incubated in growth medium containing DMSO or 50 μg/ml cycloheximide at DIV 13 for 24 h, prior to lysis in 1X SDS sample buffer. Neuronal lysates were resolved by SDS-PAGE and analysed by western blot with specific antibodies against GFP and α-tubulin. The relative abundance of pH-GluA1 (B) and pH-GluA2 (D) was calculated as the fraction of total receptors (cycloheximide/DMSO) after normalising against α-tubulin levels. Data represent mean ± S.E.M (n = 4 to 8 per group).